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GENETIC MANIPULATION OF SGT1 FOR REGULATED PRODUCTION OF α-SOLANINE IN POTATO *(Solanum tuberosum)*

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By

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Ph.D. Thesis Synopsis

Title of the Thesis	**:**	Genetic Manipulation of sgt1 for Regulated Production of α-Solanine in Potato *(Solanum tuberosum)*	
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1) Abstract

Solanum tuberosum commonly known as potato is a highly consumed non-grain crop plant that is very popular for various products. Potato has many nutritional values but has two important secondary metabolites α-Solanine and α-Chaconine. These metabolites work as a plant defence mechanism and produce a high amount of metabolites plant encounters stress. According to the researchers, when these glycoalkaloid's concentration increases to a certain level, it may cause certain neurological and gastrointestinal impairments. α-Solanine and α-Chaconine work in a co-dependent manner and have been shown to interact with Acetylcholinesterase. Acetylcholinesterase is an essential enzyme and, at the postsynaptic cleft, splits the acetylcholine-signalling molecule into acetyl + Choline; α-Solanine and α-Chaconine inhibit the acetylcholinesterase. To understand how these two glycoalkaloids bind with acetylcholinesterase. We performed molecular docking analysis to analyze at which site and to know which confirmation ligands get bind with the AChE. The analysed result showed the highest docking score of α-Solanine (-16.645 Kcal/Mol) using acetylcholine and donepezil as reference molecules. In addition, molecular dynamics simulation was also performed for figuring out confirmation changes during simulation, using Schrodinger Maestro software. As Potato is an inevitable component of every kitchen across the globe, it becomes essential to lower down the effect of these glycoalkaloids. Based on preliminary research analysis, only one glycoalkaloid α- Solanine was targeted for down-regulation without harming the plant's biological defence system. This study targets an enzyme *UDP-galactosyltransferase* that adds sugar moieties to the precursor of α-Solanine called solanidine. UDP-galactosyltransferase is coded by Sgt1 gene. Therefore; for downregulation of the α-Solanine, sgt1 was targeted using CRISPRi/Cas9. Compared to the control untransformed plant, our transgenic potato expressing the sgt1 gene in suppression mode produced a significantly lower α-Solanine level. In contrast, the level of α-Chaconine remained unchanged. Nutritional assessments of transgenic tubers revealed no significant differences in nutritional composition compared to tubers from non-transformed plants.

2) Introduction

Solanum tuberosum, widely known as potato grown over the centuries for humans, is the fifth highest non-grain consumable crop in the world. *Solanum tuberosum* belongs to the solanaceae family and has been characterized by the presence of several types of glycoalkaloids. Potatoes contain two major glycoalkaloids namely α-Solanine and α-Chaconine, derived from cholesterol through biosynthetic processes(Von Bergmann et al., 2005).

In nature, these glycoalkaloids are stress metabolites or phytoalexins that shield the plants from attacking fungi and insects. Since α-Solanine and α-Chaconine are stress metabolites, during cultivation and post-harvest handling the amplitude of these metabolites increases. The amount of glycoalkaloids depends very much on variety and regional and geographical conditions. Other factors that significantly increase the amount of glycoalkaloids are Growing Conditions, Temperature, Light, Transportation Conditions, Slicing, Sprinkling, Cutting etc. (Friedman, 2006; Nema et al., 2008). On the other hand, the concentration of glycoalkaloids in all parts of potatoes is different, such as in tuber skin, around Sprouts, around wounded areas and high concentration of alkaloids in the sprouts. In normal growth conditions, these glycoalkaloids levels are usually present in minute amount (less than 1% of the total carbon). The risk higher concentration of these glycoalkaloids gets increased when in the marketplace potatoes are exposed to sunlight. It is suggested that of 200–400 mg dose of α-Solanine and α-Chaconine can cause toxic symptoms in adult humans while 20–40 mg in children comes out with same symptom. Most commercial potatoes have a content of α-Solanine lower than 0.2 mg g−1. However, potatoes that were exposed to light and started turning green may exhibit higher concentrations(BUSHWAY et al., 1983; Bushway & Ponnampalam, 1981). α-Chachonine and α-Solanine are relatively potent inhibitors of acetylcholinesterase, an enzyme required for proper nerve synapse function. Severe cases of intoxication including deaths resulting from central nervous system depression with α-Solanine and α-Chaconine have been reported. α-Solanine and α-Chaconine also disrupt the red blood cell membranes and other cellular membranes that cause gastrointestinal and hemorrhagic symptoms. Typical potato glycoalkaloid poisoning symptoms include both gastrointestinal (abdominal pain, nausea, diarrhoea, and vomiting) and neurological symptoms (apathy, somnolence, mental confusion, dyspnoea, rapid and weak pulse, low blood pressure, and, in severe cases, coma and death)(Cantwell, 1996; Omayio et al., 2016).

α-Solanine and α-Chaconine are potent inhibitors of acetylcholinesterase enzyme, computational technique helps us to know that how the α-Solanine binds to the acetylcholinesterase, such as with peripheral active site amino acids or with core active site amino acid and this data help to gain knowledge on mechanic details of inhibition of enzyme by such alkaloids. Molecular docking techniques are aimed at predicting the best possible match Binding ligand mode to Protein (orientations / conformances like poses of the ligand within the protein binding site). Therefore, the availability of a three-dimensional molecular structure Target is a necessary condition; experimentally, it can be Structure solved (such as X-ray crystallography or NMR, Modelling homology) or structure obtained by computational techniques(Salmaso & Moro, 2018).

Due to its toxicity and teratogenicity in humans, α-Solanine was of much concern. Report indicated that several children died in Britain in the year 1899 and in 1922. Several deaths of individuals were associated with α-Solanine poisoning (Willimott, 1933). This is not the only case of multiple α -Solanine poisoning in many countries. The synorogenic effect of α-Solanine and α-chachonine on the human body remains uncertain. Various cooking types have different effects on glycoalkaloids, α -Solanine is heat stable and decomposes between 260°C and 270°C (Cárdenas et al., 2015). Consumption of raw and processed potatoes is noteworthy for having different levels of α-Solanine influence.

There are three SGA genes in potatoes that encodes for various galactosyltransferase enzymes which adds sugar moieties to the base of steroidal alkaloids and make steroidal glycoalkaloids. Sgt1 which codes for *Solanidine galactosyltransferase* add moieties of galactose to the steroidal alkaloid and make α-Solanine (Ginzberg et al., 2009).

CRISPR/Cas system is a tool that is used in Bacteria and Archaea as a part of their defence mechanism. Eventually this tool is being used for precise gene editing by number of research groups across the globe. As it is important to preserve minimal level of glycoalkaloid within potatoes for their survival after being encountered in some form of stress, in this study we have used CRISPRi / Cas9 technique, CRISPRi is strands for clustered regularly intraspaced palindromic repeats and I is for interference and Cas9 for CRISPR-associated protein 9. For CRISPR based editing, one needs guide RNA (gRNA) to complement gene of interest by guiding dCas9 (dead Cas9) protein to block transcription and gRNA (Kampmann, 2018; Mushtaq et al., 2018; Roy et al., 2018). In order to suppress α-Solanine, we have suppressed

the sgt1 gene which codes solanidine galactosyl transferase protein that eventually adds adds sugar moieties in the steroidal alkaloid structure that results to and α-Solanine production.

3) Objectives of the study

- To standardise CRISPR-Cas9 system for the introduction of SGT1 in potato system
- Transformation of Agrobacterium with CRISPR-Cas9 harbouring SGT1
- Transformation of potatoes with transformed agrobacterium
- Analysis of Transformed potato tuber for α-Solanine level
- Validation of SGT1 expression

4) Materials and Methods

To meet the adjectives narrated above, following approaches have been made:

Docking analysis and Molecular Dynamic Simulation

Molecular docking is used to evaluate and examine how α-Solanine interacts with acetylcholinesterase.

Protein preparation

Crystalline structure of acetylcholinesterase obtained from RCSB protein databank (PDB ID:-4m0e), were imported into Maestro – workspace (Schrodinger release 2018-1). Preparation of proteins was performed using OPLS3 force field. Prepared protein was analysed using Ramachandran plot. Prepared protein were used for docking and Molecular dynamic simulation studies (Harder et al., 2016; Kim et al., 2016; Sastry et al., 2013).

Ligand preparation

Using ligprep tool of Schrodinger software five ligands were prepared α-Solanine, α-Chaconine, Dehydrotomatine, Acetylcholine and Donepezil a acetylcholinesterase inhibitor all ligands were imported from Pubchem and specific chiralities were retained and force field OPLS3 was applied (Harder et al., 2016).

Induced Fit Docking

For all five ligands docking was carried out using induced fit induced-fit maestro Schrodinger, Ligand File was uploaded and amino acid residues were selected from site map

generated site that cover catalytic tried and peripheral amino acids of AChE. All other parameters were set as default (Halgren, 2007; Sherman et al., 2006).

Molecular Dynamic Simulation

For molecular dynamic simulation, simulation time was set on 50ns, temperature at 300 k, pressure at 1.01325 (Bars), and force field were OPLS3 keeping all other parameters as default. Finally molecular interaction diagram was generated and results were analysed (Bowers et al., 2006; Harder et al., 2016).

α-Solanine suppression

Vector Selection

Binary vector with pGreen vector backbone has been chosen to suppress the sgt1 gene in potatoes, because vector backbone is relatively small in size, enabling an effective expression of cas9 and gRNA in plants. The binary vector pHSN6I01 CRISPR / dCas9 pGreen-like vector has been selected (Xing et al., 2014).

Designing the gRNAs

sgRNA is designed based on the selection of target sequence of sgt1 gene of *Solanum tuberosum* obtain from https://www.ncbi.nlm.nih.gov/nucleotide/ site. Many tools are available online for the sgRNA designing, for basic design of the sgRNA are obtained from CrisprDirect and for reducing the off-target effects BLAST was done (Naito et al., 2015; Sayers et al., 2010; Ye et al., 2006).

Three different gRNA from solanum tuberosum's sgt1 gene are as follows,

1. gagggaaaaggtaattggaa
2. tatctagaacaagagggaaa
3. aaagagctatcgtttctctc

Restriction digestion

Restriction digestion was carried out by (Thermo Fisher Scientific: ER0291) ready to use kit and follow the product protocol.

Ligation

Ligation reaction was performed as described in (GBiosciences-RE-319) kit protocol with some modifications.

Competent Cell Preparation, Transformation and Confirmation of *E.coli*

Competent cell was prepared as described in (Chang et al., 2017)with minor modification, and Transformation protocol was adopted from the (Kamens, 2015) with some modifications. Confirmation of E.coli using vector specific primers.

Competent Cell Preparation, Transformation and Confirmation of *Agrobacterium tumefaciens*

Competant cell preparation and transformation of modified strain of *A.tumefaciens* (EHA105) were adopted from the (Höfgen & Willmitzer, 1988) with some modifications. Confirmation was done by Colony PCR using same vector specific primer as used for confirmation of *E.coli*.

Sterilization of Explants and Transformation of Potato Plant

Sterilization of potato sprouts (Explants) were performed as mentioned in the (Koleva Gudeva et al., 2012) with some modifications. Transformation and cultivation were performed as mentioned in (Chronis et al., 2014) with some modifications.

RNA Extraction and cDNA Synthesis

RNA extraction was carried out by using TAKARA-740949.5 product protocol and cDNA synthesis was performed as mentioned in the TAKARA-6110A product protocol.

Semi-Quantitative and RT-qPCR Analysis of F0 Plants

Semi-quantitative analysis was carried out using normal PCR conditions and intensity analysis of bands were performed using ImageJ software as described in the (Natale et al., 2011) with minor modification. RT-qPCR analysis was carried out using PCR conditions that we were used during normal PCR with modification and followed (Roche: 06402712001) product protocol. Normalize the C_T value using actin as a reference gene and analyse fold change analysis.

Temperature Shock Analysis

Temperature shock analysis was performed under specific temperature fluctuations.

F1 generation and RT-qPCR Analysis

First generation potato plants were grown in natural environment and RT-qPCR was performed with using same set-up and materials that were used for analysing F0 generation plants.

Nutritional Analysis of Transformed Potatoes

Nutritional Analysis of transformed potatoes and controlled potatoes was carried out by UV-spectrophotometer and ELISA ready to use food testing kit. These two techniques were included in AOAC (Official method of Analysis) (Performed by MODI Laboratory).

5) Results

Results are divided in two chapters

Chapter: I

Docking and Molecular Dynamic Simulation

Potato, a highly consumed non grain food across the globe produces steroidal glycol-alkaloids against environmental stress conditions. Two major Glycoalkaloids produced in potato are α-Solanine and α -Chaconine. Both of the two Glycoalkaloids are harmful to the human beyond the certain level of consumption. Excess consumption of potato leads to several neuro-physiological disorders. It has been shown that all these Glycoalkaloids are significant inhibitors of the acetylcholinesterase – an enzyme involved in neuronal signaling. To better understand the impact of glycolalkaloids on neural disorders, analysis of the inhibition capacity, conformational changes and protein ligand binding of glycoalkaloid with acetylcholinesterase becomes essential. Interactions were analyzed of a key glycoalkaloid α-Solanine with acetylcholinesterase(Dvir et al., 2010; Silman & Sussman, 2005; Xu et al., 2017). ***Docking studies with different glycoalkaloids with acetylcholinesterase showed high docking Score (-16.630 Kcal/Mol) for α-Solanine***. To better understand how Glycoalkaloids binds to the acetylcholinesterase***, Molecular dynamics simulation studies were performed and results suggested that among all glycoalkaloids taken for the simulation and docking studies, α -Solanine has been shown to form more stable Protein-ligand interaction with acetylcholinesterase that leads to disruption of neural signaling via acetylcholine***.

Chapter-II

Suppression of α-Solanine

Minimization in the level of these glycoalkaloids within tuber to reduce toxicity remains most important challenge. CRISPRi-Cas9 was used for the suppression of *Solanidine galactosyltransferase (sgt1) gene that* catalyzes solanidine to Solanine conversion during biosynthesis of α-Solanine. In the potato genome, the steroidal glycoalkaloids' (SGA) gene cluster was located at chromosome 12 (Itkin et al., 2013). We have decided to knock-down the sgt1 gene rather than knocking it out because knocking down will retain plant's defence system up to certain extent that may enable plants to survive under moderate perturbations. Bioinformatics techniques were used in this study, and gRNAs were selected from the 100 bp UTR region because CRISPRi/Cas9 showed more successful inhibition from this region(Chen et al., 2019). For designing of gRNAs sgt1 sequence was obtained from the https://www.ncbi.nlm.nih.gov/nucleotide/ site. All steps were performed that were required for the cloning and gave us positive results for cloning. Media preparation and plant transformation were successfully done. According to (McCue et al., 2005), down-regulation of SGT1 by RNAi caused α-Solanine inhibition with compensatory increases in α-Chaconine, resulting in no overall change in total glycoalkaloid concentration. In this study we suppressed the α-Solanine level in the Desiree potato using CRISPRi/Cas9 gene-editing tool.

While using the CRISPRi/Cas9 in our study, we intended to suppress α-Solanine level without disturbing the cellular concentration of α-Chaconine. Our developed transgenic potato expressing sgt1 gene in suppression mode came out with considerably low level of α-Solanine while α-Chaconine level remained unchanged in comparison to control-untransformed plant. Nutritional analyses of transgenic tubers showed no significant change in nutritional composition with tubers derived from untransformed plants.

6) Discussion

In our study we have analysed the tuber nutritional values and the results showed low level of α-Solanine and minute less in the level of α-Chaconine, where we also analysed some other nutritional values such as vitamin C content and fat content that were found to stay in normal level despite regulation of sgt1 gene. When the first generation plants were grown into the field, control as well as transgenic plants were survive in around 24º C to 32º C

temperature. Suggesting that transgenic potatoes would be a good and healthier option for human kind.

7) Summary and Conclusions

Results obtained from computational analyses suggest that the stability of the α-Solanine-Acetylcholinesterase complex is more prominent. Therefore, presence of high amount of α-Solanine in the body due to consumption of potato products leads to neural mediated toxicological effects. As potatoes are a highly consumed and staple non-grain crop across the globe, minimization in the level of these glycoalkaloids within tuber to reduce toxicity remains most important challenge. When the sgt1 gene was suppressed α-Solanine level was suppressed by approximately 80% with the 3rd gRNA and the α-Chaconine level was suppressed by approximately 20%. Suppression of sgt1 with CRISPRi/Cas9 does not upregulate α-Chaconine level and maintains the suppression of both glycoalkaloids.Nutritional analyses of transgenic tubers showed no significant change in nutritional composition with tubers derived from untransformed plants.

8) References

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9) Paper Published/Communicated

- Rathor S., **Bhatt R.**, Joshi S.; Applications of CRISPR-Cas Technology in Agriculture and associated challenges In;CRISPR/Cas System In Translational Biotechnology (Communicated)
- Pandey S.S, **Bhatt R. T**., Tiwari B.S. (2020) Plant Death: Short and Long Life Span to Immortality In; Sensory Biology of Plants Ed. Sapory S.K.
- Bhatt R**.**, Tiwari B.S. (2022) Molecular Docking and Molecular Dynamic Simulation of Steroidal Glycoalkaloids with Acetylcholinesterase (Under review)
- Bhatt R.,Tiwari B.S. Suppression of sgt1 gene by CRISPRi/Cas9 for regulated production of α-Solanine in *Solanum tuberosum* (Submitted Plant biology journal)

10) Conference/symposia Attended And Participated

- Participated in " Training on High Performance Computing and Deep Learning" held by C-DAC and GUJCOST in September 2021.
- Attended "Online Training on Image Processing Using OpenCV Python" Held on February 2021.
- Attended the webinar on "Can India Solve its Farmer's Plight and become Food Bowl of the world?" held by Biotechnika in 2020.
- Participated in EndNote certification series in august 2020 held by Clarivate.
- Participated in ISCBC-NIPiCON 2020 held at Nirma university and won best poster presentation award.
- Attended "CRISPR CONGRESS 2019" held by Biotecnika in 2019.
- Participated in workshop on microscopy held by Institute of advanced research on April 2019.